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The Combined Impact of pH and Activated Carbon on the Elemental Composition of Plant Tissue Culture Media

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Abstract

This study considered the elemental composition of plant tissue culture media in response to pH and two different types of activated carbon (tissue culture and non acid-washed grades) in liquid media. Over the pH range from 4 to 7, copper and zinc adsorbed onto the two physically different activated carbons to the same extent. Evidence suggests that copper and zinc adsorbed as hydrated inorganic species rather than as EDTA-chelates. As pH exceeded 5.8, precipitation became pronounced resulting in 50% reductions in manganese and iron and smaller reductions in calcium (20%), and phosphorous (15%), independent of AC. No adsorption was indicated for inorganic anions. Non acid-washed activated carbon released significant levels of magnesium (65 % increase) and calcium (10 % increase) at pH 5.8 compared to the control. Low levels for Cu and Zn are a concern when using AC, and low levels of Fe and Mn when the pH of the medium exceeds 5.8.

Key words: tissue culture activated carbon elemental composition

Introduction

Activated carbon (AC) is often added to plant tissue culture medium with beneficial results. In experiments conducted at IPST, different rates of culture success resulted when using two different types of activated carbon in otherwise similar media. Using different ACs may result in different nutrient compositions, perhaps explaining the observed differences in culture success. This paper is the second in a series investigating the impact of different ACs on the nutrient composition of tissue culture media.

Phan and van Staden (1998) recently reviewed the use of charcoal in vitro. Previous studies have shown the potential influence of AC on the mineral composition of tissue culture media (Van Winkle et al, 2001, companion paper this journal). The possible release of inorganic material has been investigated (Weatherhead et al. 1979) and adsorption of iron-chelates has been reported (Haberle-Bors 1980). However, other researchers found that the adsorption of chelated iron from culture medium only occurred when organic medium components were absent (Van Winkle et al, 2001, companion paper this journal). Additional literature suggests that AC is capable of adsorbing many different metallic species including cadmium, copper, zinc (Ferro-Garcia et al. 1988; Rubin & Mercer 1987; Chang & Ku 1995), nickel, lead (Corapcioglu, Huang 1987; Wilczal, Keinath 1993; Reed, Nonavinakere 1992) cobalt (Howard 1988), chromium (Bautista-Toledo et al. 1994), Fe-EDTA (Johansson et al. 1990), cyanide (Adams 1991; Kongolo et al. 1997; Petersen, Van Deventer 1997) and others. Both anions and cations are known to adsorb onto AC (Mattson, Mark 1971).

The precipitation of mineral nutrients is well-documented for Murishige and Skoog media (Teasdale 1987). The pH dependence of this precipitation has also been reported (Dalton 1983). It is known that living tissue cultures alter the pH of the medium (Bonga, Von Aderkas 1992). Therefore, it may be non-representative to study chemical composition of media at a single pH. This paper investigates the elemental composition of a tissue culture medium over an extended pH range as a function of AC type.

Experimental

Materials

The media were formulated from tissue-culture grade reagents supplied through Sigma, with the exception of casamino acids (Difco). Media were formulated using Nanopure deionized water.

Two different grades of AC were used. These were supplied by Sigma as untreated powder (C-5260), designated "N" type, and acid-washed, tissue-culture tested powder (C-9157), designated "T" type. Two production lots, designated "1" and "2" for each type were characterized.

pH Measurement

Measurements of pH were conducted using a standard pH meter and gel-filled electrode (Orion). Typically, alloquots were collected by pipette and measured in polypropylene test tubes. Titration measurements were collected directly from the titration vessels.

Activated Carbon Characterization

Ash %, point of zero charge, and the apparent surface area for the ACs used in this research are described in Van Winkle et al, 2001, companion paper this journal. Acid titration (0.01N HCl) of AC was performed using an auto-titrator (Metrohm Dosimat 665) set to a flow rate of 0.1 mL/min at room temperature (ca. 21^{o}C) and recorded by strip chart. The beaker, titrant dispensing wand, and pH probe were sealed with parafilm and a nitrogen sparge applied to the head space.

Media Preparation

The liquid media were based on the media for Norway spruce culture reported by Verhagan and Wann (1989), a modified ½ strength Brown and Lawrence (BLG) formulation (Amerson et al. 1985) with pH adjusted to 5.8. Media and explants were prepared per detailed procedures given elsewhere (Van Winkle 2000, Van Winkle et al, 2001, companion paper this journal). Cultures were grown from a single mature embryo and gametophyte (Picea abies L.S., F.W. Schumaker Company, Inc.).

The media formulations are presented in Table 1.

Table 1.

Cultures were grown on ten milliliters of media dispensed to 15 milliliter polystyrene petri plates and sealed with parafilm. Support to the growing cultures was provided by a polyester pad/ black mixed cellulose ester membrane (47 millimeter) combination.

Elemental Analyses

Elemental analyses were performed using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin Elmer Optima 3000DV) per the same procedure given in our previous paper (Van Winkle et al, 2001, companion paper this journal). Data were obtained in triplicate over a pH range of ca. 4.0 to 7.0.

The chloride and nitrate levels for liquid media were determined using capillary ion electrophoresis (Water Capillary Ion Analyzer) with fused silica capillaries and a high mobility electrolyte.

Results and Discussion

The pH of media with AC was measured two days after autoclaving, a typical timeframe prior to the introduction of the explant tissue (Figure 1). Media formulated with different ACs, T1 and N1, were significantly different in pH. The pH varied depending on how much time was allowed for pH adjustment prior to autoclaving.

Figure 1.

When pH adjustment was limited to approximately 12 minutes, the post-autoclave pH of media with N1 routinely exceeded 6.5, media with T1 gave 5.8, and control media (without AC) were near 5.6. Living Norway spruce tissues were found to drive the pH of control media and AC media with T1 to 4.8 over a period of about twenty days (Figure 2). The pH of media with N1 did not decline as rapidly and remained above 5.5 after 21 days. The elemental composition of media containing the two different ACs was therefore studied over the pH range from below 4.8 to above 6.5.

Figure 2.

Direct HCl titration

The pH response of the cultures was consistent with acid titration results for two ACs (Figure 3). The primary interest was in the region near pH 5.8 where it can be seen that the acid consumption and slopes were significantly different for these two carbons.

Figure 3.

A prominent drift in the titration end-point was observed for N1 (not shown): within fifty-five minutes of reaching pH 4.8 the pH had drifted beyond 5.5. An additional milliliter of titrant was required to again reach the end point (pH 4.8). The end-point drift was much more pronounced for N1 than for T1, indicating that the phenomenon was related to the carbon type rather than the titration conditions.

Different explanations for the slow consumption of acid by AC have been proposed in the literature. Diffusion of protons is not believed to be limiting as protons are transported through aqueous media extremely rapidly, perhaps through a very rapid transfer mechanism whereby protons are "exchanged" between adjacent water molecules. One possible explanation is that the anion, Cl^- in this case, is adsorbed and the proton accompanies it to maintain charge neutrality (Mattson, Mark 1971). Accordingly, the adsorption rate would be limited by the diffusion of hydrated Cl^-. Leon Y Leon et al. (1992) have suggested that the resistance to Cl^- diffusion arises from the charge repulsion at pore entrances and also from the diffusion through accessible pores.

Alternately, the hydrolysis of oxidized metal species may be responsible for the drift. Following activation, it is expected that much of the inorganic content of the AC exists in the form of oxides. The hydrolysis of these oxides results in the formation of hydroxide species (Cotton, Wilkinson 1976). These are basic compounds that dissociate to neutralize protons. In this case, the slow hydrolysis could be explained by slow dissolution. For media with N1, an increase in magnesium and calcium was observed and was consistent with the metal oxide hydrolysis mechanism. Acid washing would be expected to remove a significant portion of these oxides and may help to explain why acid washed carbons did not display the same degree of end-point drift.

The slope of the trace at any given point is a measure of buffering strength. The buffering strength (ΔpH/meq H^+) at pH 5.8 for T1, T2, N1, and N2, were -138, -138, -24.2, -23.1, respectively. The non-acid-washed carbons, N1 and N2, were nearly equal and displayed six times the buffering strength of T1 or T2 at pH 5.8. At pH 5.3, the non-acid washed

carbons had thirty-nine times the buffering strength of the tissue-culture grade carbons. A highly buffered medium may be a source of stress to a growing culture.

Elemental Composition: Cu, Zn, Fe, Mn

The elemental composition of the medium was found to change at a very slow rate after the initial two days; therefore, measurements at two days were treated as equilibrium data. The elements displaying the most dramatic behavior in response to either AC or pH were Fe, Mn, Zn, and Cu. The available concentration (ppm) of each element was determined from the solution phase at different pH levels. The resulting data for copper and zinc are presented as a function of medium pH for each carbon type, N1 and T1, in Figures 4 and 5.

The samples, labeled "Con 1", were media without AC, but with elevated copper and zinc levels, and included elevated hormones and vitamins (Table 1, left column). "Con 2" media did not contain AC and were formulated with normal hormone, vitamin, copper, and zinc levels (Table 1, right column). The calculated value appears as a dotted line across the figure, is based on the "Control 2" composition, and has been corrected to reflect a volume loss of 4.5% during autoclaving (Van Winkle et al, 2001, companion paper this journal).

Figure 4.

Figure 5.

Each data marker in these figures represents a single observation but most conditions were replicated at least twice and the experiments were performed over a period of months using different stock solutions to formulate the media. The variability of our results is expected to represent what may be encountered in standard practice. Many of

the replicates overlapped when plotted: the difference (range) was generally less than 3% of the mean value. However, the variability of the copper data was greater than this level, with the range sometimes exceeding 10% of the mean. The higher variability for copper was attributed to the lower levels present and hence a greater impact due to impurities introduced during sample preparation prior to analysis, and instrument variability.

Copper and zinc (Figures 4 and 5) were the only mineral elements present in the medium for which significant adsorption onto AC was measured. It is expected that cobalt was also adsorbed, but the concentrations of cobalt were below the detection threshold of the instrument. Cobalt adsorption onto AC has been documented (Howard, 1988). However, cobalt complexes have lower stability constants than those for copper and zinc (Morel, Hering, 1993); thus, cobalt adsorption was not expected to significantly impact the adsorption of the other two ions. In any event, cobalt is not considered to be an essential nutrient (Minocha, 1987), and so its probable depletion is not a critical concern.

As shown in Figure 4, approximately 95% of the copper was adsorbed (compare Con 1 with AC-containing media, T or N). Nearly 50% of the zinc was adsorbed for media below pH 5.8. Compensating for adsorption by doubling the initial concentration of zinc and increasing copper twenty fold, resulted in copper and zinc levels which were in good agreement with those for media without AC (compare "Con 2" to "T", "N " data), confirming prior results (Pullman et al., 1995). Note that the present data do not indicate significant differences based on AC type, suggesting that adsorption was limited by a factor other than the physical character of the two ACs.

Zinc and copper differed in pH response. For control media without AC (Con 1 and Con 2), zinc declined in concentration as pH increased. For media with N1 and T1 the data were inconclusive; however, if the data points at pH 4.0 are neglected, a pH response was indicated for N1. Copper, showed no pH trends for either control media, but showed slightly increased availability with increased pH for the media containing AC. Differences in copper levels were insignificant for the media with the different AC's.

Elemental Composition: Fe

Data are presented for iron in Figure 6. Notice that the available iron level did not approach the calculated amount at any pH. The iron level demonstrated strong pH dependence, decreasing more than 50% over the range from pH 4.8 to pH 6.8. The pH response was very similar to that noted for zinc. It may be seen that the media with AC were in very good agreement with the control (Con 2) and with each other: adsorption was insignificant.

Figure 6.

Elemental Composition: Mn

Data for manganese vs. pH are shown in Figure 7. It is apparent that Mn declined in response to pH changes to a degree that exceeded iron (Fe^{3+}) losses, and over a narrower pH range. The control medium responded in a similar manner to media containing AC: no adsorption of manganese was indicated. The data were similar for media with the different AC types.

Figure 7.

Remaining Elements: B, K, S, P, Ca, Mg, Cl, NO_3

Of the remaining nutrients, boron (as boric acid), sulfur (present as sulfate), and potassium showed no dependence on either AC or pH. Phosphorus and calcium, however, showed a pH dependence above pH 6.5 with decreased availability of about 20% and 15%, respectively (Figures 8 and 9). Data for the anions, Cl^- and NO_3^-, were collected using capillary ion electrophoresis. Neither anion was adsorbed onto AC.

Figure 8.

Figure 9.

For media containing N1, both calcium and magnesium were released into the medium resulting in 10% and 65% increases, respectively. Increased pH led to a decline in available magnesium of 20% (Figure 10). The difference in calcium concentration between media with N1 and the control was insignificant above pH 6.5, indicating that a solubility product had been achieved. Phosphorous, calcium, and magnesium all exceeded the calculated levels in medium contaiing AC N1. Casamino acids were calculated to contribute approximately 1.75 ppm of phosphorous when 500 mg/l were added to medium. Van Winkle et al 2001(companion paper this journal) demonstrated that N1 contributed significant amounts of Ca (+16%) and Mg (44%) when added at 1.25 g/l. Silicon was the only inorganic impurity consistently released at significant levels into the media with AC. Molybdenum appeared to be depleted through precipitation to about 60% of the calculated level independent of pH or the presence of AC.

Figure 10.

For media without AC, the formation of a precipitate in Murishige and Skoog-based media is a well-known phenomenon that has been primarily attributed to the precipitation of iron as ferric phosphate (Teasdale 1987). The results from this study indicate that the elemental composition of that precipitate is a function of the medium pH. It was found that the molar percentage of calcium present in the precipitate doubled (from ca. 16% to 33%) as the pH increased from 5.8 to 6.5. The molar percentage of phosphorus increased by only 2% (37 to 39%) while magnesium increased by more than a factor of 2-3%. The flocc-like appearance of the precipitate and its pronounced onset at pH 6.5 after

autoclaving suggested the formation of hydroxide species that probably incorporated phosphorus (Elliott 1994).

Reversible Precipitation

Norway spruce, loblolly pine and Douglas-fir cultures tend to drive down the pH of growth media. A precipitate formed at pH 5.8 may be dissolved at pH 4.8. To test whether the precipitation was reversible, media without AC were formulated, adjusted to pH 6.8 and autoclaved. Samples were collected and analyzed. The media were readjusted to pH 4.8 and allowed to equilibrate at room temperature overnight prior to sampling. Control samples were formulated and autoclaved at pH 4.8. Referring to Figure 11, the left bar for each ion represents the medium at pH 6.8 (normalized to the control value); the right bar represents the same media after adjustment to pH 4.8. The data at pH 6.8 show that Fe, Mg, Mn, P, and Zn decreased relative to the pH 4.8 control, but there was no difference for copper. After adjustment of the medium to pH 4.8, Mg, Mn, P, and Zn were completely recovered. A percentage of the iron (18%) remained insoluble. The complete recovery of phosphorus indicated that a portion of the iron was precipitating in a non-phosphate form.

Figure 11.

Summary of AC and pH Effects

A summary of the elemental composition of media with the two different carbons is presented in Table 2 below. The composition of media with N1 reflects the elevated pH, as well as the release of ions (Mg, Si).

Table 2.

Conclusions

Different elemental compositions may result when using different ACs in tissue culture media. Adsorption and pH effects were of primary interest. Copper and zinc were the only elements in the medium to adsorb (ca. 95% and 50%, respectively) onto AC. The level of adsorption of these elements onto two different carbons was similar, suggesting that their adsorption was limited by factors unrelated to carbon character. The data suggest that copper and zinc did not adsorb as EDTA-chelated species. Instead, these two elements were probably adsorbed selectively as hydrated ions and also possibly as co-adsorbates with other organic molecules.

The elemental composition of tissue culture medium was primarily determined by the pH of the medium, with Mn and Fe decreasing by more than 50% over the pH range from 4.8 to 6.8. Calcium and zinc also displayed a significant decline as pH increased to 6.5. Carbon type (acid-washed tissue culture grade vs. non-acid-washed) influenced pH. Hence, the main concern, with respect to the inorganic elemental composition, when using different ACs is their impact on the pH of the medium.

When using non-acid-washed ACs, impurities may be introduced into the medium. The non-acid-washed AC of this study introduced into the medium about 10% more calcium, 65% more magnesium, and 30% more Si than were present for control media at pH 5.8. Non-acid-washed carbon proved difficult to neutralize due to its high buffering strength. Therefore, non-acid washed AC may be a poor choice for tissue culture medium.

Assuming that the pH of the growth medium remains below 5.8, the primary concern regarding the mineral composition when using AC is the depletion of copper and zinc.

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Figure Legends

Figure 1. Medium pH 2 days after preparation. Media were adjusted to pH 5.8 prior to addition of AC and 0, 12, or 35 minutes after addition of 1.25 g/L AC. T1 and N1 designate tissue culture-grade activated carbon (Sigma C-9157) and untreated activated carbon (Sigma C-5260), respectively. Error bars depict the 90% confidence interval based on three replications.

Figure 2. Medium pH over time in the presence of tissue. Media contain 1.25 g/L N1 or T1 AC or no AC (Con 2). Data points represent pH measurements from single culture plates.

Figure 3. Acid titration curves for N1 and T1. HCl (0.01N) was dispensed at 0.1mL/min into AC slurry (0.25g per 100mL degassed, nanopure H_2O). Headspace was sparged with nitrogen during titration.

Figure 4. Available copper vs. activated carbon type and pH. "Con 1" refers to media with elevated Cu (20x), Zn (2x), hormones, vitamins, and no AC. "Con 2 " refers to media with normal Cu, Zn, hormones, vitamins, and no AC. AC media (N1 or T1 at 1.25 g/L) contain Cu (20x), Zn (2x), elevated hormones and elevated vitamins. Error bars depict the 90% confidence interval based on three replications.

Figure 5. Available zinc vs. activated carbon type and pH. See also Figure 1 legend.

Figure 6. Available iron vs. activated carbon type and pH. See also Figure 1 legend.

Figure 7. Available manganese vs. activated carbon type and pH. See also Figure 1 legend.

Figure 8. Available phosphorous vs. activated carbon type and pH. See also Figure 1 legend.

Figure 9. Available calcium vs. activated carbon type and pH. See also Figure 1 legend.

Figure 10. Available magnesium vs. activated carbon type and pH. See also Figure 1 legend.

Figure 11. Reversibility of precipitation by pH change. Media were prepared at pH 6.8, autoclaved, and sampled after one day. Media readjusted to pH 4.8, equilibrated overnight, and sampled again. Values are averages of two replications and have been normalized to those obtained previously for media at pH 4.8.

Tables

Table 1a. Comparison of liquid culture media formulations*.

Mineral components

Component	AC and Control 1	Control 2
KNO_3	50	50
KH_2PO_4	85	85
KCl	372.5	372.5
$CaCl_2 * 2H_2O$	220	220
$MgSO_4 * 7H_2O$	160	160
KI	0.415	0.415
H_3BO_3	3.1	31
$MnSO_4 * H_2O$	8.45	8.45
$ZnSO_4 * 7H_2O$	8.6	4.3
$Na_2MoO_4 * 2H_2O$	0.125	0.125
$CuSO_4 * 5H_2O$	0.25	0.0125
$CoCl_2 * 6H_2O$	0.0125	0.0125
$FeSO_4 * 7H_2O$	13.9	13.9
Na_2EDTA	18.65	18.65

*Liquid: Modified ½ BLG (Verhagen, Wann 1989)

Table 1b. Comparison of liquid culture media formulations, Organic components.

Component	AC and Control 1	Control 2
Sucrose	10,000	10,000
myo-Inositol	50	50
Casamino acids	500	500
L-Glutamine	750	750
Thiamine HCl	0.15	0.05
Pyridoxine HCl	0.15	0.05
Nicotinic Acid	0.75	0.25
L-Asparagine	50	50
2,4-D	100	2
BAP	90	1
Activated carbon	0;1250	0
pH	5.8	5.8

Liquid: Modified ½ BLG (Verhagen, Wann 1989)

Table 2. Available elemental composition of media with AC

Ion	N1, pH 6.5*	T1, pH 5.8*
Cu	0.003	0.004
Zn	0.8	0.95
Fe	1.1	2.25
Mn	1.6	2.7
B	0.67	0.67
S	26.6	26.6
K	330	330
P	18	21.8
Ca	58	60
Mg	21.3	17
Co	n/a	n/a
Mo	0.03	0.03
Si	1.8	1.05

*Values are given in ppm; initial Cu, Zn elevated by 20x and 2x to compensate for AC adsorption.

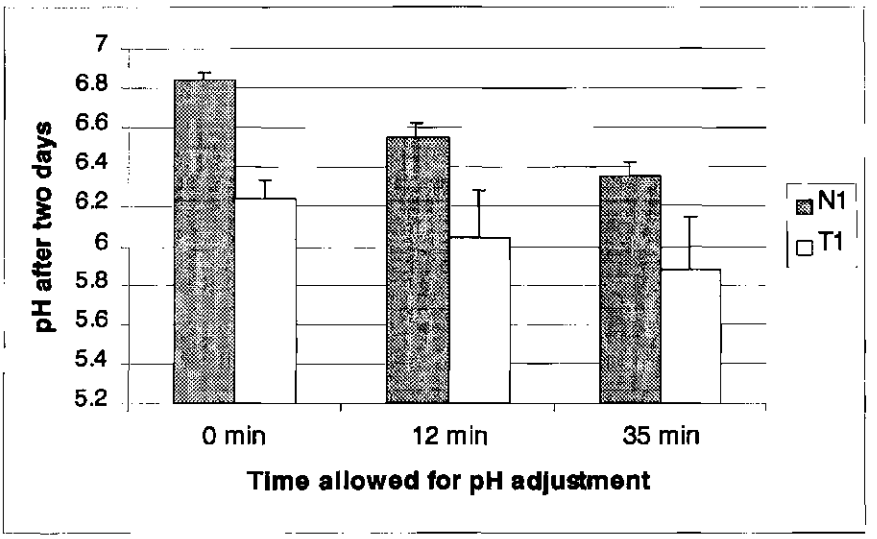


Figure 1.

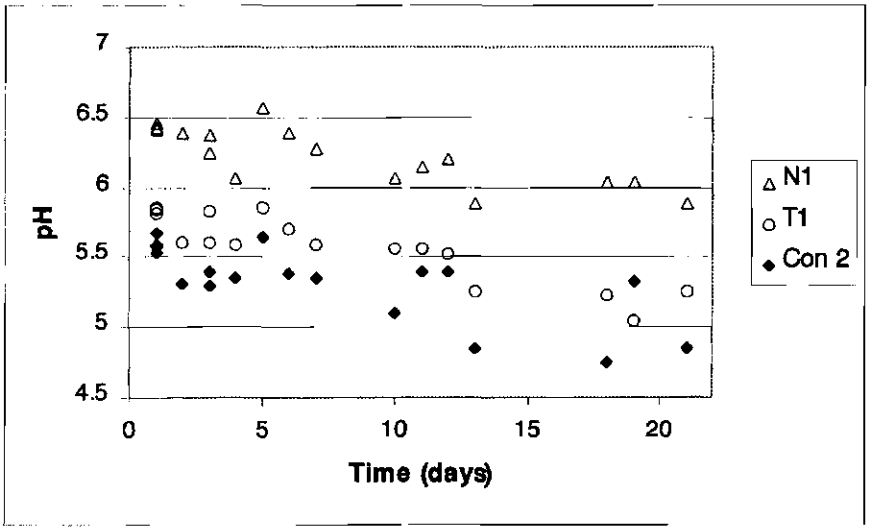
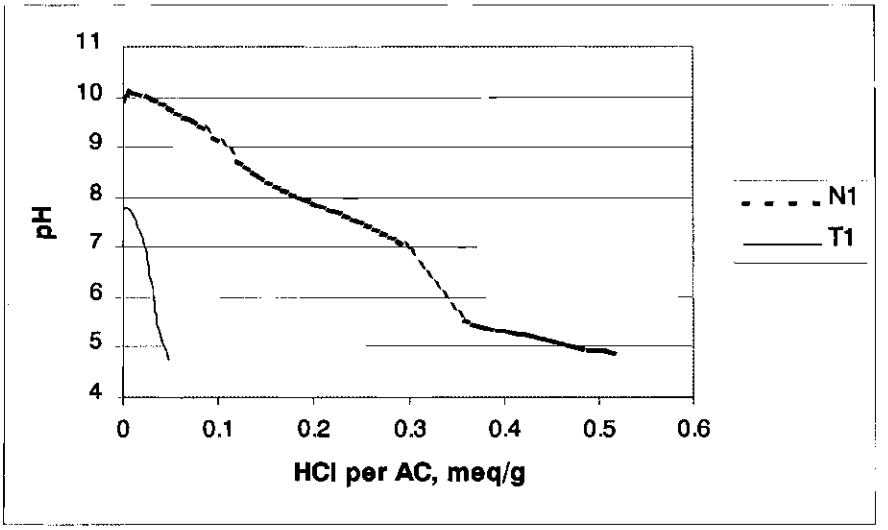


Figure 2.



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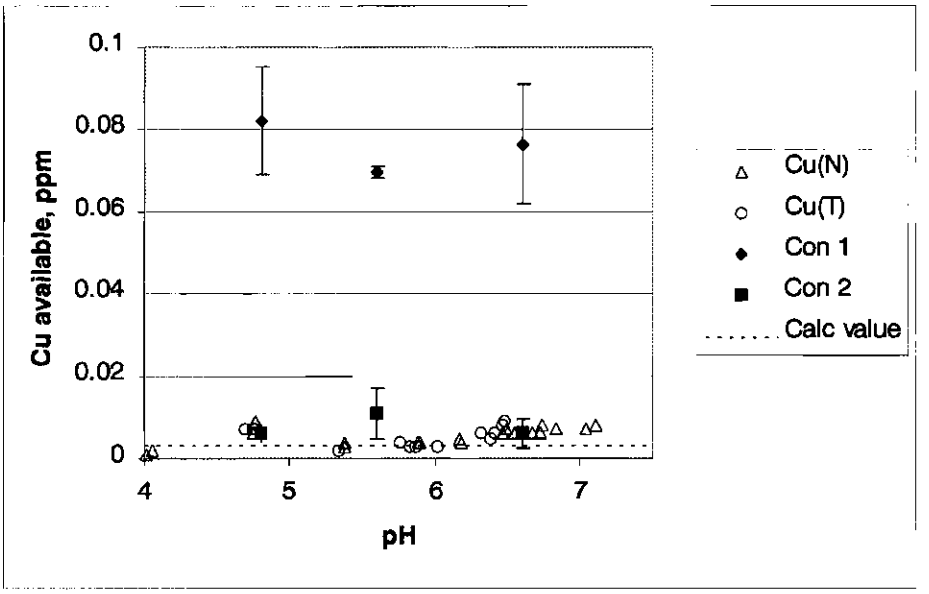

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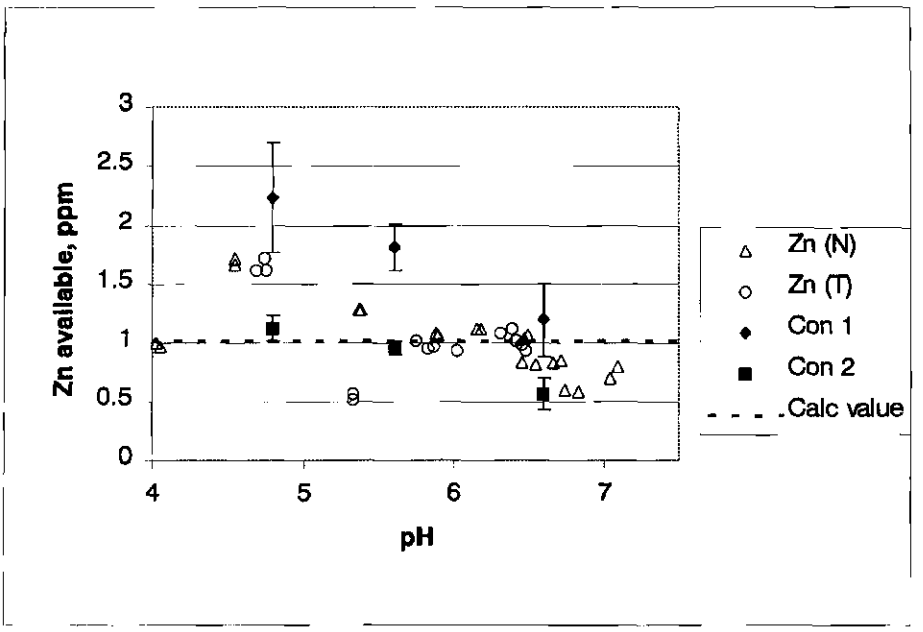

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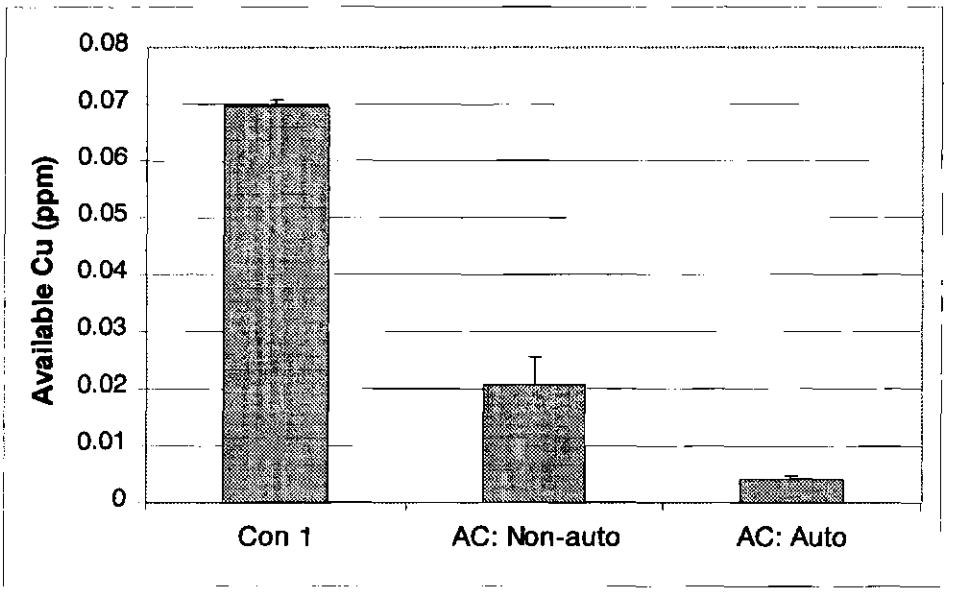

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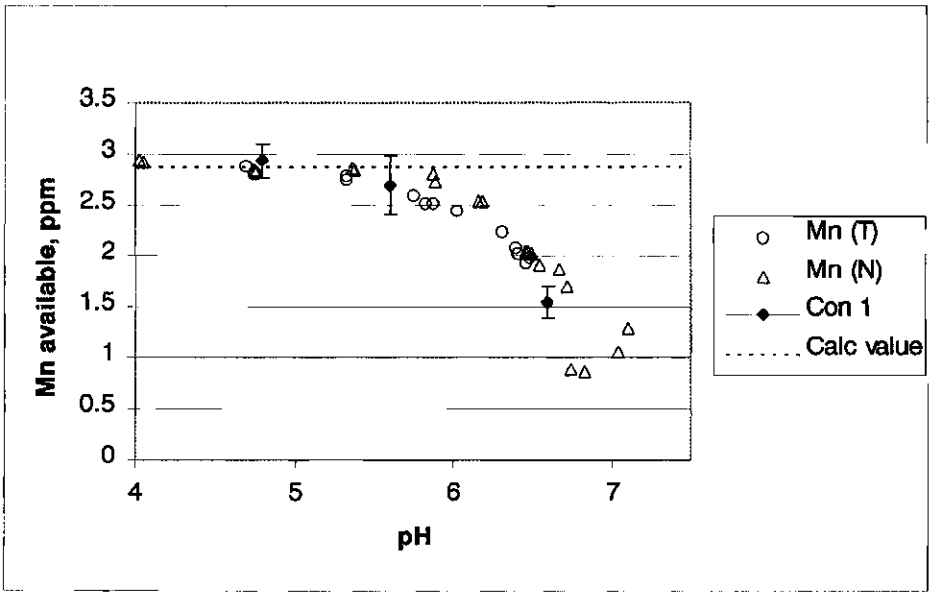

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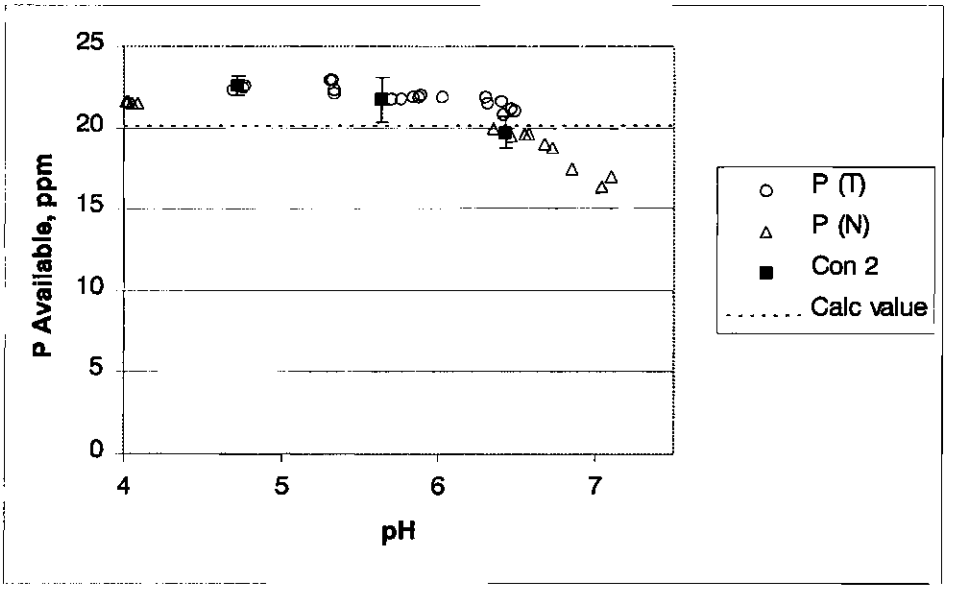

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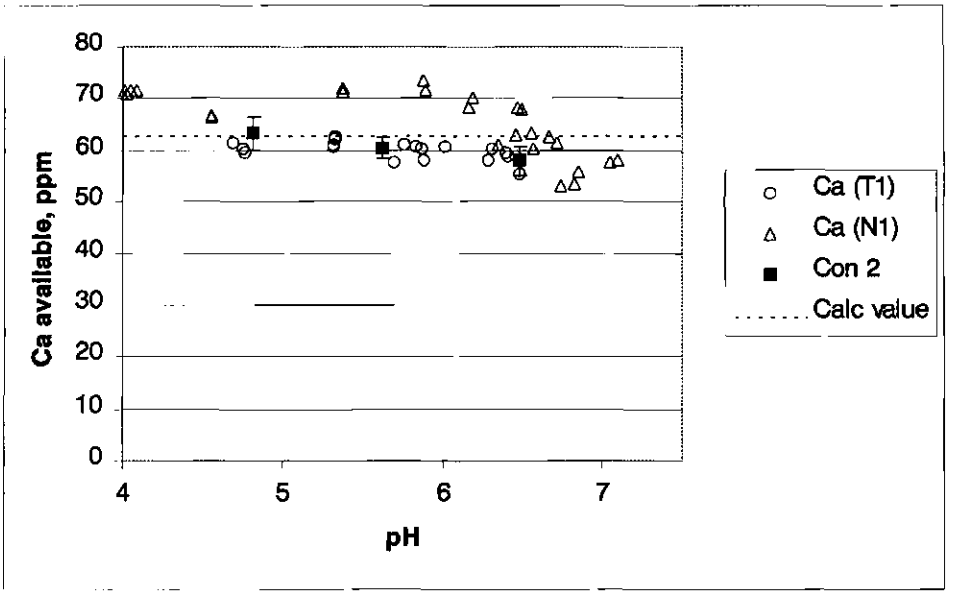

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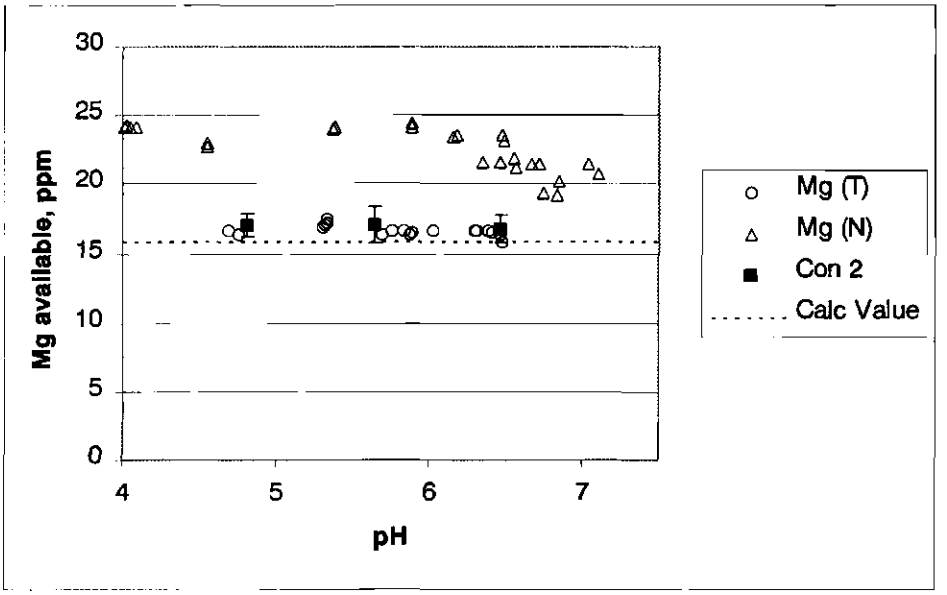
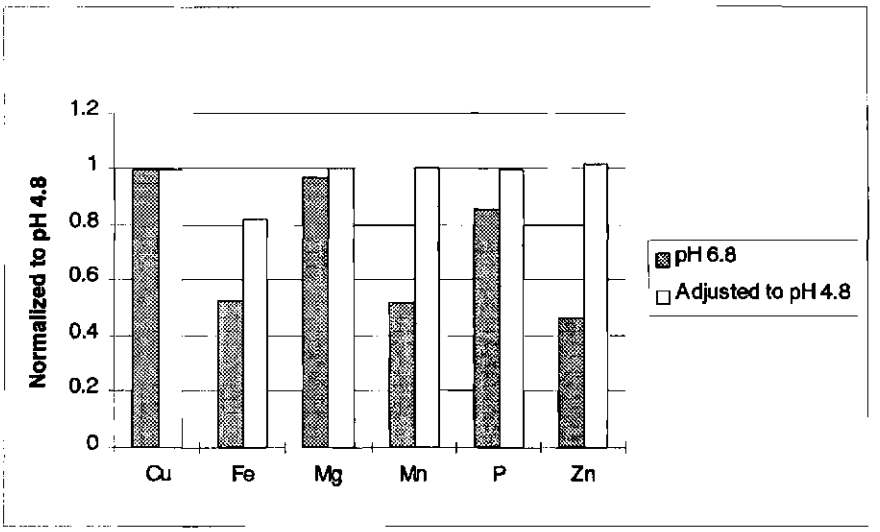


Figure 10.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1
0.8
0.6
0.4
0.2
0
Normalized to pH 4.8
Cu
Fe
Mg
Mn
P
Zn
pH 6.8
Adjusted to pH 4.8
Figure 11.